Enrichment of FLI1 and RUNX1 mutations in families with excessive bleeding and platelet dense granule secretion defects

Stockley, J.; Morgan, N. V.; Bem, D.; Lowe, G. C.; Lordkipanidze, M.; Dawood, B.; Simpson, M. A.; Macfarlane, K.; Horner, K.; Leo, V. C.; Talks, K.; Motwani, J.; Wilde, J. T.; Collins, P. W.; Makris, M.; Watson, S. P.; Daly, M. E.

DOI:
10.1182/blood-2013-06-506873

blood

2013 122: 4090-4093
doi:10.1182/blood-2013-06-506873 originally published online October 7, 2013

Enrichment of *FLI1* and *RUNX1* mutations in families with excessive bleeding and platelet dense granule secretion defects

Jacqueline Stockley, Neil V. Morgan, Danai Bem, Gillian C. Lowe, Marie Lordkipanidzé, Ban Dawood, Michael A. Simpson, Kirsty Macfarlane, Kevin Horner, Vincenzo C. Leo, Katherine Talks, Jayashree Motwani, Jonathan T. Wilde, Peter W. Collins, Michael Makris, Steve P. Watson and Martina E. Daly

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Brief Report

PLATELETS AND THROMBOPOIESIS

Enrichment of *FLI1* and *RUNX1* mutations in families with excessive bleeding and platelet dense granule secretion defects

Jacqueline Stockley,[1] Neil V. Morgan,[2] Danai Bem,[2] Gillian C. Lowe,[2] Marie Lordkipanidzé,[2] Ban Dawood,[2] Michael A. Simpson,[3] Kirsty Macfarlane,[2] Kevin Horner,[4] Vincenzo C. Leo,[1] Katherine Talks,[5] Jayashree Motwani,[6] Jonathan T. Wilde,[7] Peter W. Collins,[8] Michael Makris,[1] Steve P. Watson,[2] and Martina E. Daly,[1] on behalf of the UK Genotyping and Phenotyping of Platelets Study Group

[1]Department of Cardiovascular Science, University of Sheffield Medical School, University of Sheffield, Sheffield, United Kingdom; [2]Centre for Cardiovascular Sciences, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom; [3]Division of Genetics and Molecular Medicine, King's College London School of Medicine, Guy's Hospital, London, United Kingdom; [4]Department of Coagulation, Royal Hallamshire Hospital, Sheffield, United Kingdom; [5]Department of Haematology, Royal Victoria Infirmary, Newcastle Upon Tyne, United Kingdom; [6]Department of Haematology, Birmingham Children's Hospital, Birmingham, United Kingdom; [7]Adult Haemophilia Centre, Queen Elizabeth Hospital, Birmingham, United Kingdom; and [8]Arthur Bloom Haemophilia Centre, School of Medicine, Cardiff University, Cardiff, United Kingdom

Key Points

- Novel *FLI1* and *RUNX1* alterations were identified in 6 of 13 patients with excessive bleeding and platelet granule secretion defects.
- Two *FLI1* alterations predicting amino acid substitutions in the DNA-binding domain of FLI1 abolished transcriptional activity of FLI1.

We analyzed candidate platelet function disorder genes in 13 index cases with a history of excessive bleeding in association with a significant reduction in dense granule secretion and impaired aggregation to a panel of platelet agonists. Five of the index cases also had mild thrombocytopenia. Heterozygous alterations in *FLI1* and *RUNX1*, encoding Friend leukemia integration 1 and RUNT-related transcription factor 1, respectively, which have a fundamental role in megakaryocytopoeisis, were identified in 6 patients, 4 of whom had mild thrombocytopenia. Two *FLI1* alterations predicting p.Arg337Trp and p.Tyr343Cys substitutions in the FLI1 DNA-binding domain abolished transcriptional activity of FLI1. A 4-bp deletion in *FLI1*, and 2 splicing alterations and a nonsense variation in *RUNX1*, which were predicted to cause haploinsufficiency of either FLI1 or RUNX1, were also identified. Our findings suggest that alterations in *FLI1* and *RUNX1* may be common in patients with platelet dense granule secretion defects and mild thrombocytopenia. (*Blood*. 2013; 122(25):4090-4093)

Introduction

Inherited platelet function disorders (PFDs), which cause excessive bleeding, are heterogeneous and can seldom be linked to a causative gene using clinical and laboratory phenotype alone.[1] The advent of techniques allowing selective capture and enrichment of target gene sequences, coupled with next-generation sequencing (NGS) technology, has facilitated the rapid identification of disease-associated variants by enabling the simultaneous analysis of large numbers of genes. We have used NGS to investigate index cases from 13 unrelated families recruited to the UK Genotyping and Phenotyping of Platelets (UK GAPP) study[2] with significantly reduced platelet dense granule secretion using lumi-aggregometry.

Study design

Recruitment of patients and platelet phenotyping

Index cases and affected relatives from a subgroup of 13 families, recruited to the UK GAPP study on the basis of excessive clinical bleeding and a suspicion of an inherited platelet disorder, were investigated. These individuals were diagnosed with a platelet dense granule secretion defect following platelet phenotyping but had no other features of Hermansky-Pudlak syndrome. The inclusion and exclusion criteria for recruitment to the study, and the criteria for diagnosis of a dense granule secretion defect, have been described.[3] Platelet aggregation and adenosine triphosphate (ATP) secretion were assessed in platelet-rich plasma using a rationalized panel of platelet agonists and a dual-channel Chronolog lumi-aggregometer.[3] This study was approved by the National Research Ethics Service Committee West Midlands–Edgbaston (REC reference: 06/MRE07/36) and conducted in accordance with the Declaration of Helsinki.

Analysis of candidate genes

Genomic DNA was isolated from peripheral blood, and NGS of candidate PFD genes was undertaken on an ABI-SOLiD3+ or an Illumina Genome Analyzer IIx platform.[2,4] Novel sequence variations identified by NGS were confirmed by conventional Sanger sequencing. Where possible, patients were selected for NGS analysis on the basis of having an affected relative who was also available for study.

Submitted June 4, 2013; accepted September 18, 2013. Prepublished online as *Blood* First Edition paper, October 7, 2013; DOI 10.1182/blood-2013-06-506873.

J.S. and N.V.M. contributed equally to this study.

The online version of this article contains a data supplement.

There is an Inside *Blood* commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Table 1. Genotypic and phenotypic characteristics of subjects with platelet dense granule secretion defects

Family (F)/patient identification*	*FLI1* or *RUNX1* alteration†	Effect	Platelet count ($\times 10^9$/L)‡	Mean platelet volume (fL)	ATP secretion (nmol/1 $\times 10^8$ platelets)§
F1; II.1	*FLI1*; c.1009 C>T	p.Arg337Trp	244	10.3	0.37
F1; III.1	*FLI1*; c.1009 C>T	p.Arg337Trp	180	10.5	0.07
F2; II.5	*FLI1*; c.1028 A>G	p.Tyr343Cys	92	8.8	ND‖
F2; III.1	*FLI1*; c.1028 A>G	p.Tyr343Cys	117	8.6	ND‖
F3.1	*FLI1*; c.992-995del	p.Asn331Thrfs*4	142	11.4	0.38
F3.2	*FLI1*; c.992-995del	p.Asn331Thrfs*4	157	11.8	0.57
F4.1	*RUNX1*; c.508+1 G>T	Splicing	302	7.3	0.32
F4.2	*RUNX1*; c.508+1 G>T	Splicing	70	7.5	ND
F4.3	*RUNX1*; c.508+1 G>T	Splicing	130	7.1	0.62
F5	*RUNX1*; c.351+1G>T	Splicing	139	8.0	0.35
F6.1	*RUNX1*; c.317 G>A	p.Trp106Stop	100	8.0	0.25
F7	–	–	233	8.5	0.57
F8	–	–	190	8.5	ND
F9	–	–	205	8.4	0.48
F10	–	–	370	8.6	0.31
F11	–	–	225	NA	0.63
F12	–	–	245	NA	0.52
F13	–	–	114	10.9	0.12

Heterozygous nucleotide changes present in *FLI1* and *RUNX1* and their predicted effects on the resulting RNA or protein are shown.

NA, not available; ND, not detectable.

*Index cases are indicated in bold font.

†Alterations are numbered according to positions in the NM_002017.3 and NM_001754 transcripts for *FLI1* and *RUNX1*, respectively.

‡Mean platelet counts are shown, the normal reference range is 150×10^9 to 400×10^9 platelets per L, and thrombocytopenia is defined as platelet count $<150 \times 10^9$ platelets per L.

§ATP secreted in response to 100 μM of PAR-1 receptor–specific peptide SFLLRN; fifth percentile in healthy volunteers is 0.82 nmol/1 $\times 10^8$ platelets.

‖ATP secretion was measured in response to 1 U/mL of thrombin in the center where these subjects were recruited, against a normal reference range of 0.73 to 1.80 nmol/1 $\times 10^8$ platelets.

Detection of MYH10 in platelets

Nonmuscle myosin heavy chain IIB (MYH10) was detected in platelets by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting as described.[5]

Results and discussion

To date, 366 patients, from 292 unrelated families, have been recruited to the UK GAPP study, of whom 56 (15%) have been classified as having a secretion defect. NGS analysis of candidate PFD genes was undertaken in 13 of the patients with defects in secretion, who were selected, where possible, on the basis of having an affected relative who was also available for study. Novel heterozygous alterations in either the *FLI1* or *RUNX1* gene were identified in 6 of the families (F1 to F6) (Table 1). Two nonsynonymous *FLI1* alterations, c.1009C>T and c.1028A>G, predicting p.R337W and p.Y343C substitutions in the highly conserved DNA-binding domain of FLI1, were identified in affected members of F1 and F2, and a 4-bp frameshift deletion in *FLI1* (c.992-995del; p.Asn331Thrfs*4) was present in both affected members of F3. Three *RUNX1* variations predicted to cause RUNX1 haploinsufficiency were identified; donor splice site transversions, c.508+1G>T and c.351+1G>T, were detected in F4 and F5, respectively, and a nonsense mutation in codon 106 (c.317G>A; p.Trp106Stop) was detected in the index case from F6.

The presence of an *FLI1* or *RUNX1* alteration was associated with symptoms of excessive bleeding in all index cases, and the patients' affected family members, and with mild thrombocytopenia in 5 of the families (Table 1; supplemental Figure 1, see the *Blood* Web site). In F4, the splice site alteration in *RUNX1* was associated with a normal platelet count in the index case, but with thrombocytopenia in 2 affected relatives. The missense variations in *FLI1* were also associated with alopecia, eczema or psoriasis, and recurrent viral infections in affected individuals in F1, and with mild thrombocytopenia, alopecia, and eczema in the affected individuals in F2 (Figure 1A-B).

Examination of platelet secretion and aggregation in all subjects carrying *FLI1* or *RUNX1* defects consistently revealed the predominant platelet abnormality to be a significant reduction in platelet ATP secretion in response to all agonists tested (Figure 1C, Table 1, supplemental Figure 2, and data not shown). As reported previously for patients with dense granule secretion defects, reductions in platelet aggregation in response to collagen and PAR-1 were observed in some, but not all, subjects and were usually more apparent at low or intermediate agonist concentrations (data not shown).[3]

FLI1 is a member of the ETS (E–twenty six) family of transcription factors, which is expressed primarily in hematopoietic cells and regulates genes expressed both early and late in megakaryocytopoiesis, including *GP6*.[6,7,8-10] Patients with Paris-Trousseau syndrome (PTS), who are hemizygous for *FLI1* as a result of a deletion of chromosome 11q23, have an increased tendency to bleed, which is associated with thrombocytopenia, and enlarged platelets displaying giant α-granules.[10-12] It is thought that transient monoallelic expression of *FLI1* at an early stage during megakaryocytopoiesis, coupled with hemizygous loss of *FLI1* in patients with PTS, which results in a complete lack of FLI1 expression, accounts for the generation of 2 distinct subpopulations of normal and immature megakaryocytes in patients with PTS.[10] The identification of alterations predicting substitutions in the DNA-binding domain of FLI1 and their association with symptoms in 2 families suggests a role for FLI1 in the pathogenesis of bleeding in these families.

We investigated whether the p.R337W and p.Y343C substitutions could alter the DNA-binding capacity of FLI1 by examining the ability of the recombinant FLI1 variants to bind to an ETS site in the *GP6* promoter using the dual-luciferase reporter assay to measure

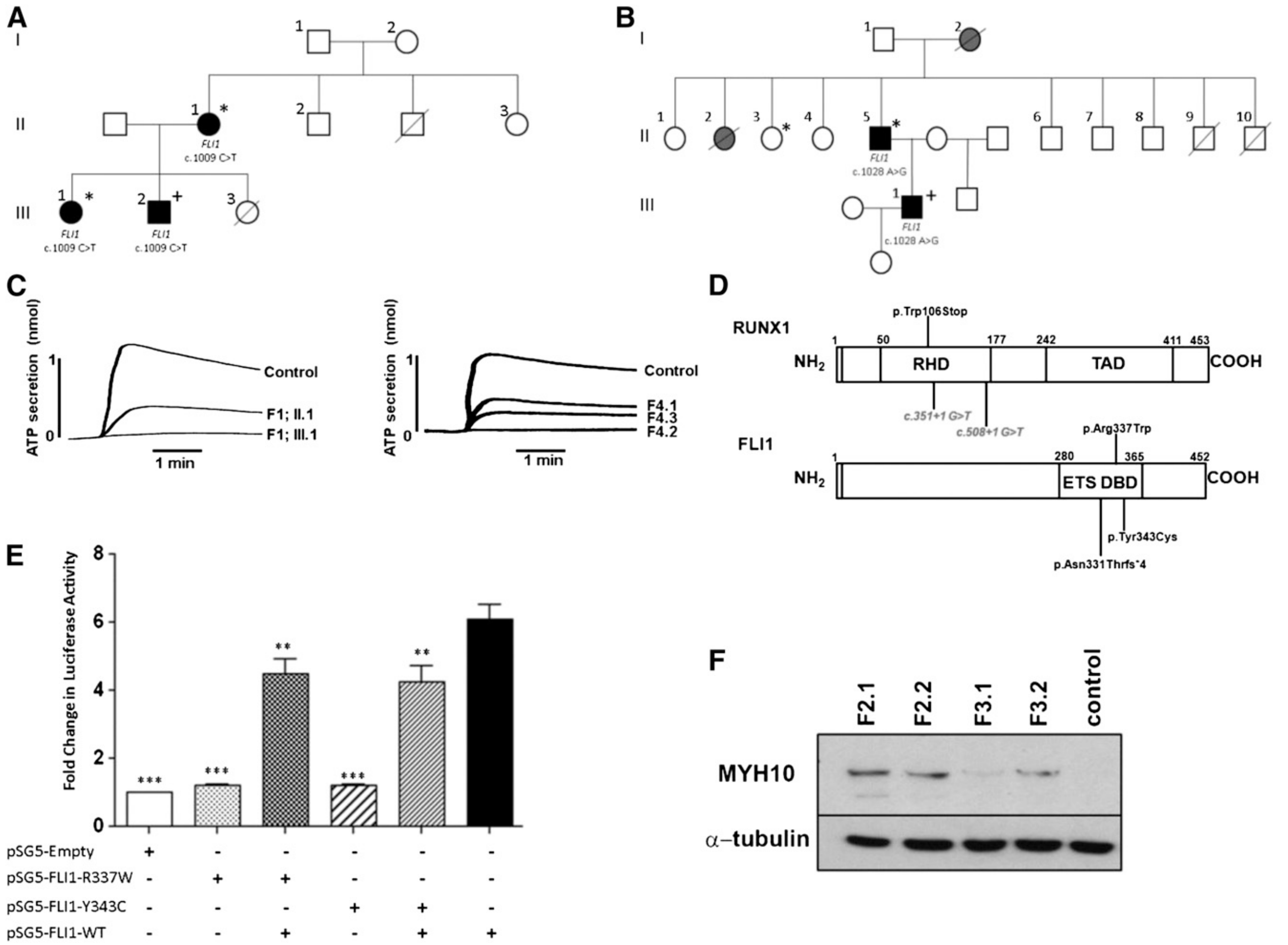

Figure 1. Excessive bleeding and platelet dense granule secretion defects are associated with heterozygous mutations in *FLI1* and *RUNX1*. (A-B) Pedigrees showing inheritance of mild bleeding, alopecia totalis, and other clinical features in families 1 (A) and 2 (B). Individuals heterozygous for the c.1009C>T and c.1028A>G transitions in *FLI1* are indicated. Lines through symbols indicate deceased individuals. In panel A, individuals with bleeding symptoms, alopecia, and confirmed platelet dense granule secretion defects are indicated by black filled symbols. An asterisk indicates the presence of eczema and a history of recurrent viral infections. The presence of psoriasis is indicated by a "+" sign. In panel B, individuals with bleeding symptoms and alopecia are indicated by black or gray filled symbols. Black filled symbols indicate individuals with confirmed platelet dense granule secretion defect and mild thrombocytopenia. An asterisk indicates a history of infective endocarditis, and the presence of eczema and colitis is indicated by a "+" sign. (C) ATP secretion in response to 100 μM PAR1 peptide in 2 members of F1 with the c.1009C>T transition in *FLI1* and 3 members of F4 with the c.508+1G>T transversion in *RUNX1* alongside controls. (D) Schematic diagram of RUNX1 and FLI1 showing the regions of the proteins affected by mutations identified in this study. Intronic mutations, predicted to interfere with splicing of the *RUNX1* RNA, are shown in italics. (E) HEK293T cells were cotransfected with wild-type (WT) and variant FLI1 constructs, or combinations thereof, and pGL3.10-GP6-luciferase and pRLnull-Renilla reporters as shown, and firefly and Renilla luciferase expression assessed in cell lysates 48 hours later. Data represent the means (± standard error of the mean) of 3 independent experiments; $^{**}P < .01$; $^{***}P < .001$. (F) MYH10 protein expression in platelets from patients with *FLI1* mutations (F2.1 and F2.2 with p.Tyr343Cys FLI1 mutation; F3.1 and F3.2 with p.Asn331Thrfs*4 FLI1 mutation) and a healthy control.

GP6 promoter activity. There was a complete loss in the ability of the R337W and Y343C FLI1 variants to induce *GP6* promoter activity compared with that of WT-FLI1 ($P < .001$; Figure 1E). Furthermore, coexpression of either the R337W or the Y343C variant with WT-FLI1, to mimic heterozygosity, resulted in significant reductions in the transcriptional activity to ~60% of that of WT-FLI1 alone ($P < .01$), indicating that these mutations disrupt DNA binding of FLI1 and will cause a reduction in activation of megakaryocyte-specific genes (Figure 1E). Of interest is a previous study, which showed that R337 is critical for the function of nuclear localization signal 2 of FLI1 and that an R337A substitution caused a reduction in nuclear accumulation of FLI1.[13] However, as with the R337W variant, the R337A substitution was shown to disrupt the DNA-binding ability of FLI1 and, consequently, to downregulate expression of FLI1-inducible megakaryocyte-specific genes.[13]

As a member of the RUNT family of transcription factors, the role of RUNX1 in regulating megakaryopoiesis and platelet formation is well established. RUNX1 has been shown to cooperate with FLI1 in the late stages of megakaryopoiesis.[14,15] Inherited *RUNX1* mutations are recognized to lead to autosomal dominant thrombocytopenia and impaired platelet function and are associated with a propensity to myelodysplastic syndrome and acute myeloid leukemia.[1] RUNX1-mediated silencing of the *MYH10* gene is required for the switch from mitosis to endomitosis during megakaryocyte maturation. The persistence of MYH10 in platelets was recently proposed as a biomarker for *RUNX1* alterations in patients with familial thrombocytopenia and for *FLI1* deletions in PTS.[5,16] The detection of MYH10 in platelets from patients carrying the p.Tyr343Cys and the p.Asn331Thrfs*4 *FLI1* variations confirms the use of MYH10 detection as a biomarker for *FLI1* alterations (Figure 1F). Indeed, immunoblot detection of MYH10 expression in platelets would be a useful initial screening test for inherited platelet disorders caused by abnormalities in *RUNX1* or *FLI1*. Dosage of *RUNX1* is thought to contribute to the risk of AML because missense mutations that result in variants that can heterodimerize with normal RUNX1 and act in a dominant-negative manner to compete with the normal protein have been reported to be associated with a higher risk of hematologic malignancy than mutations resulting in haploinsufficiency.[17] The absence of hematologic malignancies to date in the 3 families with *RUNX1* mutations identified in this study, which were all predicted to result in haploinsufficiency, would support this observation.

The identification of *RUNX1* or *FLI1* alterations in 6 of 13 unrelated index cases with dense granule secretion disorder, 4 of whom had thrombocytopenia, suggests that defects in these genes will account for a significant proportion of patients with defects in platelet granule secretion and excessive bleeding.

Acknowledgments

The authors thank the Centre for Genome Research, University of Liverpool, for enrichment of genomic DNA and NGS on the ABI-SOLiD3+ platform and Professor M. Trojanowska, Boston University, for providing the WT-FLI1 expression construct.

This work was supported by the British Heart Foundation (RG/09/007/27917) and the Wellcome Trust (093994). S.P.W. holds a British Heart Foundation Chair. The GAPP project is included in the UK National Institute for Health Research Non Malignant Haematology Specialty Group Portfolio (ID 9858) and receives support in patient recruitment from this group and from Comprehensive Local Research Networks, with Birmingham and the Black Country acting as the lead Comprehensive Local Research Network. The authors acknowledge the support of all collaborating clinicians and staff in participating UK hemophilia centers.

Authorship

Contribution: J.S. and M.E.D. wrote the paper; J.S., N.V.M., D.B., G.C.L., M.L., B.D., M.A.S., K.M., K.H., and V.C.L. contributed to the data collection and laboratory analyses; G.C.L., M.L., K.T., J.M., J.T.W., P.W.C., and M.M. recruited patients and contributed clinical data to the study; M.E.D. and S.P.W. coordinated the study; and all authors read and commented on the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Martina Daly, Department of Cardiovascular Science, University of Sheffield Medical School, Beech Hill Rd, Sheffield, S10 2RX, United Kingdom; e-mail: m.daly@sheffield.ac.uk.

References

1. Nurden AT, Freson K, Seligsohn U. Inherited platelet disorders. *Haemophilia.* 2012;18(suppl 4):154-160.
2. Watson SP, Lowe GC, Lordkipanidzé M, Morgan NV; GAPP Consortium. Genotyping and phenotyping of platelet function disorders. *J Thromb Haemost.* 2013;11(suppl 1):351-363.
3. Dawood BB, Lowe GC, Lordkipanidzé M, et al. Evaluation of participants with suspected heritable platelet function disorders including recommendation and validation of a streamlined agonist panel. *Blood.* 2012;120(25):5041-5049.
4. Cullup T, Kho AL, Dionisi-Vici C, et al. Recessive mutations in *EPG5* cause Vici syndrome, a multisystem disorder with defective autophagy. *Nat Genet.* 2013;45(1):83-87.
5. Antony-Debré I, Bluteau D, Itzykson R, et al. MYH10 protein expression in platelets as a biomarker of RUNX1 and FLI1 alterations. *Blood.* 2012;120(13):2719-2722.
6. Watson DK, Smyth FE, Thompson DM, et al. The ERGB/Fli-1 gene: isolation and characterization of a new member of the family of human ETS transcription factors. *Cell Growth Differ.* 1992;3(10):705-713.
7. Furihata K, Kunicki TJ. Characterization of human glycoprotein VI gene 5′ regulatory and promoter regions. *Arterioscler Thromb Vasc Biol.* 2002;22(10):1733-1739.
8. Hashimoto Y, Ware J. Identification of essential GATA and Ets binding motifs within the promoter of the platelet glycoprotein Ib alpha gene. *J Biol Chem.* 1995;270(41):24532-24539.
9. Bastian LS, Kwiatkowski BA, Breininger J, Danner S, Roth G. Regulation of the megakaryocytic glycoprotein IX promoter by the oncogenic Ets transcription factor Fli-1. *Blood.* 1999;93(8):2637-2644.
10. Raslova H, Komura E, Le Couédic JP, et al. FLI1 monoallelic expression combined with its hemizygous loss underlies Paris-Trousseau/Jacobsen thrombopenia. *J Clin Invest.* 2004;114(1):77-84.
11. Hart A, Melet F, Grossfeld P, et al. Fli-1 is required for murine vascular and megakaryocytic development and is hemizygously deleted in patients with thrombocytopenia. *Immunity.* 2000;13(2):167-177.
12. Favier R, Jondeau K, Boutard P, et al. Paris-Trousseau syndrome: clinical, hematological, molecular data of ten new cases. *Thromb Haemost.* 2003;90(5):893-897.
13. Hu W, Philips AS, Kwok JC, Eisbacher M, Chong BH. Identification of nuclear import and export signals within Fli-1: roles of the nuclear import signals in Fli-1-dependent activation of megakaryocyte-specific promoters. *Mol Cell Biol.* 2005;25(8):3087-3108.
14. Tijssen MR, Cvejic A, Joshi A, et al. Genome-wide analysis of simultaneous GATA1/2, RUNX1, FLI1, and SCL binding in megakaryocytes identifies hematopoietic regulators. *Dev Cell.* 2011;20(5):597-609.
15. Huang H, Yu M, Akie TE, et al. Differentiation-dependent interactions between RUNX-1 and FLI-1 during megakaryocyte development. *Mol Cell Biol.* 2009;29(15):4103-4115.
16. Lordier L, Bluteau D, Jalil A, et al. RUNX1-induced silencing of non-muscle myosin heavy chain IIB contributes to megakaryocyte polyploidization. *Nat Commun.* 2012;3:717.
17. Matheny CJ, Speck ME, Cushing PR, et al. Disease mutations in RUNX1 and RUNX2 create nonfunctional, dominant-negative, or hypomorphic alleles. *EMBO J.* 2007;26(4):1163-1175.